"METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESYLATE AND ENALAPRIL MALEATE, AMLODIPINE BESYLATE AND LISINOPRIL, AMLODIPINE BESYLATE AND LOSARTAN POTASSIUM IN TABLET DOSAGE FORM USING SPECTROPHOTOMETRY AND HPLC"

A Synopsis

Submitted in

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of

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(Pharmacy)

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INTRODUCTION

Method development is a process to develop certain methods which should be cost effective, rapid, and economic with higher repeatability and accuracy. Often this is required in case of any newly launched drugs or any new combination. [1]

Analytical methodology consist of-

- Technique
- Method
- Procedure
- Protocol [2]

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs. [3]

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for the quantitation of the drug in biological fluids may not be available.

- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable. [3]

Techniques used in Method Development:

Most important techniques fit into one of the three principal areas: Spectroscopy, Electrochemistry and Chromatography.

A] Spectroscopic Techniques.

Ultraviolet & Visible Spectrophotometry, Fluorescence & Phosphorescence Spectrophotometry, Atomic Spectrometry (emission and absorption), Infrared Spectrophotometry, Raman Spectroscopy, X-ray Spectroscopy, Radiochemical Technique including activation analysis, Nuclear Magnetic Resonance Spectroscopy, Electron Spin Resonance Spectroscopy.

B] Electrochemical Techniques.

Potentiometry, Voltametry, Volta metric Technique, Amperometric Technique, Coulometry, Electrogravimetry, Conductance Technique.

C] Chromatographic Techniques.

Gas Chromatography, High performance liquid chromatography, High performance thin-layer chromatography, Electrophoresis.

D] Miscellaneous techniques.

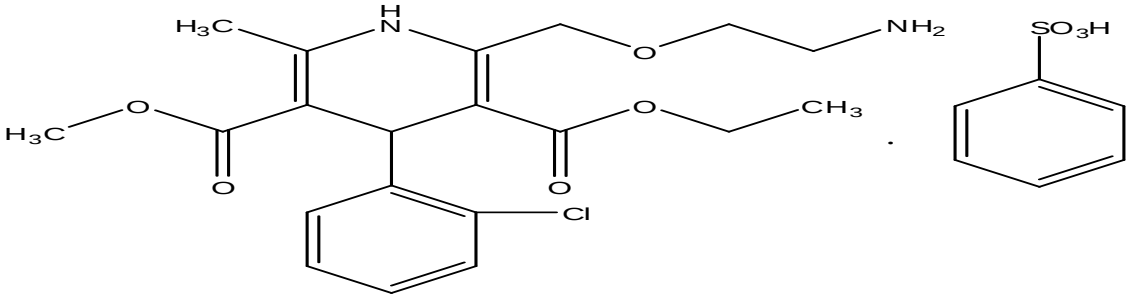
Thermal Analysis, Mass Spectrometry, Kinetic Technique.

E] Hyphenated or combined techniques.

GC-MS (Gas Chromatography- Mass Spectrometry), ICP-MS (Inductively coupled Plasma Mass Spectrometry), GC-IR (Gas Chromatography-Infrared Spectroscopy), MS-MS (Mass Spectroscopy-Mass Spectroscopy). [4]

DRUG PROFILE

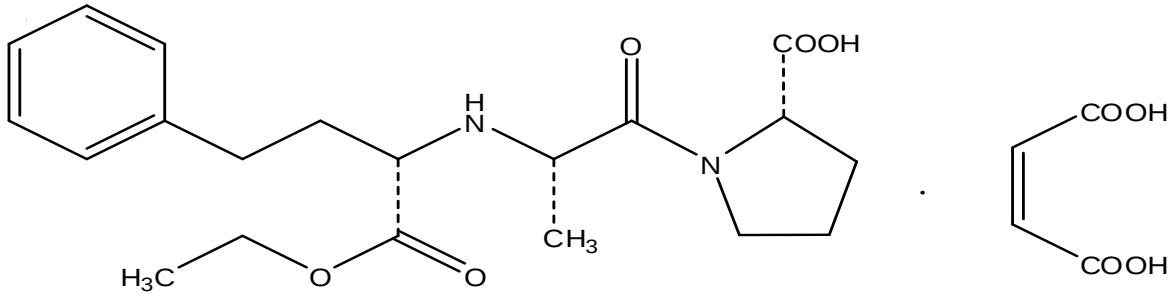
Amlodipine Besylate



- **IUPAC Name -** 3-ethyl 5-methyl (4*RS*)- 2- [(2-aminoethoxy) methyl]- 4- (2-chlorophenyl) -6-methyl- 1,4- dihydropyridine-3,5- dicarboxylate benzene sulphonate. [5]
- **Molecular formula**- $C_{26}H_{31}ClN_2O_8S$. [5]
- **Molecular weight**- 567.1. [5]
- **Solubility**-Freely soluble in methanol, slightly soluble in water and 2-propanol, sparingly soluble in ethanol. [6]
- **Pharmacodynamics**- Amlodipine act by blocking voltage-sensitive calcium channels. It acts on L-type channels. Amlodipine slow conduction in the SA and AV nodes where action potential propagation depends on slow inward Ca^{2+} current, slowing the heart and terminating SVT by causing partial AV block. It shortens the plateau of the action potential and reduces the force of contraction. Reduced Ca^{2+} entry reduces after depolarization and thus suppresses premature ectopic beats. [7]
- **Toxicity**- Headache, peripheral oedema, fatigue, somnolence, nausea, abdominal pain, flushing, dyspepsia, palpitations, dizziness. Rarely pruritus, rash, dyspnoea, asthenia, muscle cramps etc. [8]
- **Dose regimen**- *Adult:* 5mg once daily. *Elderly:* 2.5mg once daily. [8]
- **Formulations available in market**- available in tablet form named as AMDEPIN (Cadila), AMLOACT (Active HC), DEFIDIN (Lupin); and also available in

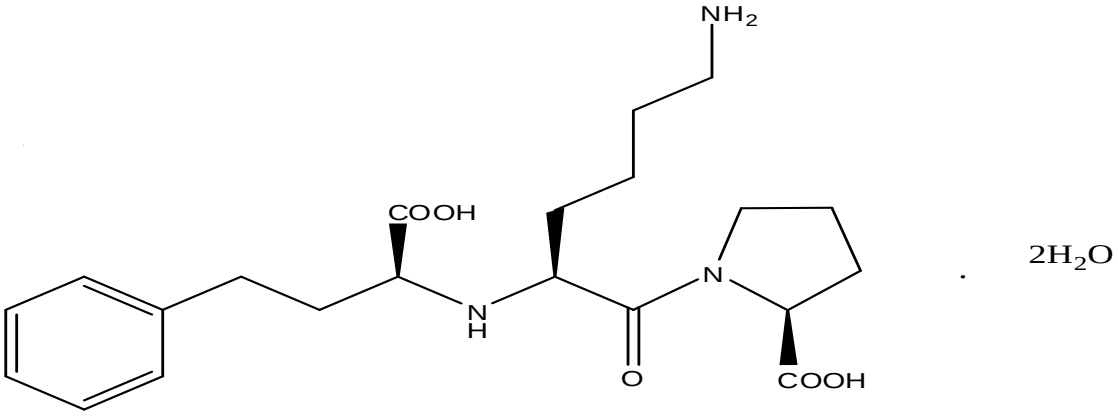
combination with enalapril as AMACE (Systopic), AMLOGEN-EL (Alkem), AMTAS-E (Intas), DILVAS-AM (Cipla). [8]

Enalapril maleate



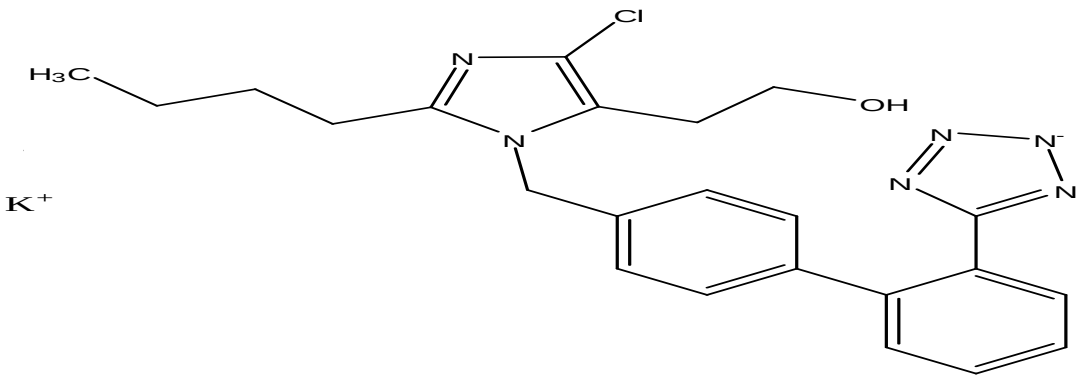
- **IUPAC Name**- (2*S*)-1-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl) 3phenylpropyl] amino] propanoyl] pyrrolidin-2-carboxylic acid hydrogen maleate[5]
- **Molecular formula**- $C_{20}H_{28}N_2O_5$, $C_4H_4O_4$. [5]
- **Molecular weight**- 492.5. [5]
- **Solubility**-Freely soluble in methanol, sparingly soluble in water, practically insoluble in methylene chloride. [6]
- **Pharmacodynamics**- *Enalapril* is a prodrug-converted in the body to *enalaprilat* (a tripeptide analogue). It abolishes the action of A-I but not that of A-II: does not block A-II receptors. It can also increase plasma kinin levels and potentiate the hypotensive action of exogenously administered bradykinin. [7]
- **Toxicity**- Initial hypotension may be severe and prolonged. Dizziness, headache, fatigue, persistent dry cough, abnormal taste, lassitude, rash, neutropenia, renal impairment or failure. [8]
- **Dose regimen**- *Adult:* 5mg once daily. *Elderly:* 2.5mg once daily. *Child:* 80mcg/kg once daily. [8]
- **Formulations available in market**- available in tablet form named as BQL (Zydus Cadila), DILVAS (Cipla), LUPINACE (Lupin); and also available in combination with amlodipine as AMACE (Systopic), AMLOGEN-EL (Alkem), AMTAS-E (Intas), DILVAS-AM (Cipla). [8]

Lisinopril



- **IUPAC Name**- (*S*)- 1-[N2-(1-carboxy-3-phenylpropyl)-L-lysyl]-Lprolinedihydrate. [5]
- **Molecular formula**- $C_{21}H_{31}N_3O_5$, $2H_2O$. [5]
- **Molecular weight**- 441.5. [5]
- **Solubility**- Soluble in water, sparingly soluble in methanol, practically insoluble in acetone and in anhydrous ethanol. [6]
- **Pharmacodynamics-** Lisinopril inhibits the action of A-I but not that of A-II: does not block A-II receptors. [7]
- **Toxicity**- Dizziness, headache, fatigue, GI and taste disturbances, persistent dry cough and other upper respiratory tract symptoms, skin rash, angioedema, photosensitivity, renal impairment, hypokalaemia, cholestatic jaundice, alopecia etc. [8]
- **Dose regimen**- *Adult:* 5-10mg once daily. [8]
- **Formulations available in market**- available in tablet form named as LIPRIL (Lupin), CIPRIL (Cipla), LISOPRIL (IPCA); and also available in combination with amlodipine as AMLACE (USV), AMLOPRES-L (Cipla), LIPRIL-AM (Lupin). [8]

Losartan Potassium



- **IUPAC Name**- 4-butyl-4-chloro-1- [[2′-(1*H*-tetrazol-5-yl)[1,1′-biphenyl]- 4-yl]methyl]-1*H*-imidazole-5-methanol. [5]
- **Molecular formula**- $C_{22}H_{22}ClKN_6O$. [5]
- **Molecular weight**- 461.0. [5]
- **Solubility**- Freely soluble in water, slightly soluble in acetonitrile, soluble in isopropyl alcohol. [6]
- **Pharmacodynamics-** Losartan is a competitive antagonist and inverse antagonist of A-II receptor. It blocks all overt actions of A-II, *viz.* vasoconstriction, release of aldosterone and Adr from adrenals, renal actions promoting salt and water reabsorption, central actions like thirst, vasopressin release and growth promoting actions on heart and blood vessels. [7]
- **Toxicity-** Headache, dizziness, back pain, myalgia, respiratory tract disorders, fatigue, rash, angioedema, neutropenia, GI disturbances, transient elevation of liver enzymes, renal impairment, hyperkalaemia. [8]
- **Dose regimen**- *Adult:* 50mg once daily. *Elderly:* 25mg once daily. *Child:* 700mcg/kg once daily. [8]
- **Formulations available in market**- available in tablet form named ALSARTAN (Aristo), COSART (Cipla), COVANCE (Ranbaxy), LOSAGARD (Lupin); and also available in combination with amlodipine as ALSARTAN-AM (Aristo), AMLOPRES-Z (Cipla), LOSACAR-A (Cadila HC). [8]

OBJECTIVES

- To develop UV and HPLC method for simultaneous estimation of Amlodipine besylate and Enalapril maleate, Amlodipine besylate and Lisinopril, Amlodipine besylate and Losartan.
- To validate the method developed using parameters like accuracy, precision, linearity range for the estimation of these drugs in pharmaceutical dosage form.

REVIEW OF LITERATURE

Amlodipine Besylate and Enalapril Maleate

1) Garg G., Saraf S.(2008) developed three simple, sensitive and specific spectrophotometric methods and validated for quantization of enalapril maleate and amlodipine besylate in tablet dosage form. In simultaneous equation method, the drugs were determined by using the absorptivity values of enalapril and amlodipine at 209 and 238 nm respectively. Second method was based on the determination of graphical absorbance ratio at two selected wavelengths; one being the isoabsorptive point of the drugs (219 nm) and the other being the absorption maxima of amlodipine (238 nm), in this method both the drugs obeyed the Beer-Lambert's law in the concentration range of 6-18 µg/ml. The third method was based on the derivative spectrophotometric method at zero crossing wavelengths.[9]

Amlodipine Besylate and Lisinopril

1) Joshi HV., Patel JK.(2011) developed two simple, accurate, precise, reproducible, requiring no prior separation and economical procedures for simultaneous estimation of Amlodipine besylate (AML) and Lisinopril (LIS) in tablet dosage form have been developed. First method was simultaneous equation method; in this method 360.0 nm and 248.0 nm were selected to measure the absorbance of drugs at both wavelengths. The second method was Q-value analysis based on measurement of absorptivity at 300.0 nm (as an iso-absorptive point) and 360.0 nm. AMD and LIS at maximum wavelength of AML, 360.0 nm and at isoabsorptive point 300.0 nm shows linearity in a concentration range of 5-40 µg/mL. Recovery studies range from >99.82% for AMD and >98.09% for LIS in case of simultaneous equation method and >100% for AMD and >98.45% for LIS in case of Q-analysis method confirming the accuracy of the proposed method.[10]

2) Rathee P., Rathee S., Thakur S., Kumar V (2010) developed a new second derivative UV-Spectrophotometric method for the simultaneous assay of amlodipine besylate and lisinopril dihydrate in bulk drug and in tablet dosage forms using double distilled water as the solvent. The method is based on

simultaneous equation or Vierodt's method. The lmax values for amlodipine besylate and lisinopril dihydrate in the solvent medium were found to be 256 nm and 216 nm respectively. The systems obey Beer's law in the range of 10.0 to 70.0 µg/ml and 4.0 to 40.0 µg/ml with correlation coefficient of 0.9994 and 0.9996 for amlodipine besylate and lisinopril dihydrate respectively. Repeatability, Interday and intraday precision were found to be 0.134, 0.280, 0.349 and 0.205, 0.530, 0.569 respectively. No interference was observed from common tablet adjuvants. t –test and F-test have been applied for the recovery studies of the method.[11]

3) Chauhan V., Prajapati ST., Patel CN.(2011) developed a reverse phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of amlodipine and lisinopril in marketed formulation. The determination was carried out on a Phenomenex C18 (250 x 4.6 mm, 5 µm) column using a mobile phase of 0.02 M phosphate buffer solution: methanol (75:25v/v, pH 7.0). The flow rate was 1.0ml/min with detection at 212 nm. The retention time for amlodipine was 4.11 min and for lisiopril 7.29 min. Amlodipine and Lisinopril showed a linear response in the concentration range of 10-110 µg/ml. The correlation co-efficient (' *r* ' value) for amlodipine and lisinopril was 0.9991 and 0.9992, respectively. The results of analysis have been validated statistically and by recovery studies. The percentage recoveries obtained for amlodipine and lisinopril ranges from 100.04 to 100.57%.[12]

4) Bhaskara VR., Rao AL.(2011) developed a simple, reproducible and efficient reverse phase high performance liquid chromatographic method for simultaneous determination of lisinopril and amlodipine in tablets. A column having 150 × 4.6 mm i.d. in isocratic mode with mobile phase containing acetonitrile: phosphate buffer (60:40; adjusted to pH 3.0) was used. The flow rate was 0.5 ml/min and effluent was monitored at 215 nm. The retention time (min) and linearity range (µg/ml) for lisinopril and amlodipine were (4.111, 3.097) and (20-60, 10-30), respectively.[13]

Amlodipine Besylate and Losartan

1) Srinivasan S. et al (2010) developed a rapid, sensitive and specific RP-HPLC method involving UV detection was developed and validated for determination and quantification of amlodipine and losartan potassium in tablet dosage form. Chromatography was carried out on a pre-packed C18; 250x4.6mm, 5µ, SS column (Kromasil) mm using filtered and degassed mixture of Buffer:Acetonitrile:Methanol (55:30:15) as mobile phase at a flow rate of 1.5ml/min and effluent was monitored at 237nm. The method was validated in terms of linearity, precision, accuracy, and specificity, robustness and solution stability.[14]

2) Patil PR. et al (2009) developed a simple, precise, accurate and reproducible reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of losartan potassium (LP) and amlodipine besylate (AB) was developed. Chromatography was carried out on RP C-18 Column (Microsorb-MV 100-5, 250 x 4.6 mm) using 0.02% triethylamine in water: acetonitrile (60:40), pH adjusted to 2.5 with O- phosphoric acid as mobile phase at a flow rate of 1.0 ml/min and the detection wavelength was 226 nm. The retention time for LP and AB was found to be 2.32 and 10.10 min, respectively. Proposed method was validated for precision, accuracy, linearity range, robustness and ruggedness.[15]

3) Patil PR. et al (2009) developed two simple, accurate, precise, reproducible, requiring no prior separation and economical procedures for simultaneous estimation of losartan potassium and amlodipine besylate in tablet dosage form have been developed. First method employs formation and solving of simultaneous equation using 208 nm and 237.5 nm as two analytical wavelengths for both drugs in methanol. The second method is Q value analysis based on measurement of absorptivity at 242.5 nm (as an iso-absorptive point) and 237.5 nm. losartan potassium and amlodipine besylate at their respective λmax 208 nm and 237.5 nm and at isoabsorptive point 242.5 nm shows linearity in a

concentration range of 2-20 µg/mL. Recovery studies range from 99.95% for Losartan Potassium and 99.33% for amlodipine besylate in case of simultaneous equation method and 102.93% for losartan potassium and 101.02% for amlodipine besylate in case of Q - analysis method confirming the accuracy of the proposed method.[16]

4) Pedroso CF et al (2009) developed a simple RP–LC method for simultaneous quantification of losartan and amlodipine and separation of their degradation products was developed. The method was validated with an ODS column. A gradient of acetonitrile and phosphate pH 3.0 buffer was utilized as mobile phase. The linearity was determined at 50–150% level. Individual recoveries at 70–130% level ranged from 98.8 to 100.5% for losartan and 96.4–101.2% for amlodipine. The robustness was also evaluated.[17]

METHODOLOGY

Characterization and Identification

a) Melting Point: The melting point of Amlodipine besylate, Losartan potassium, Lisinopril dihydrate and Enalapril maleate were determined by using melting point apparatus.

b) Solubility: In a stepwise procedure, increasing volumes of water are added at room temperature to approximately 0.1 g of the sample in a 10 ml glass stoppered measuring cylinder. After each addition of an amount of water, the mixture is shaken for 10 minutes and is visually checked for any undissolved parts of the sample. If, after addition of 10 ml of water, the sample or parts of it remain undissolved, the experiment is continued in a 100 ml measuring cylinder. When the solubility is low, a long time may be required to dissolve a substance and at least 24 hours should be allowed. If, after 24 hours, the substance is still not dissolved, more time (up to 96 hours) should be allowed or further dilution.

c) Stability of drugs:

The stability of drugs was observed by leaving drugs for few hours to check the sedimentation or degradation of the drug in the solvent.

Simultaneous equation method

Two wavelengths selected for the method λ_1 and λ_2 that are absorption maximas of Amlodipine besylate and Enalapril maleate/Lisinopril dihydrate/Losartan potassium respectively in methanol: 1N HCl (1:1) or methanol: 0.1N HCl (1:1). The stock solutions of both the drugs were further diluted separately with methanol: 1N HCl (1:1) or methanol: 0.1N HCl (1:1) to get a series of standard solutions of 0-30µg/ml concentrations. The absorbances were measured at the selected wavelengths and absorptivities (A 1%, 1 cm) for both the drugs at both wavelengths were determined as mean of three independent determinations.

Concentrations in the sample were obtained by using following equations-

$$C_x = \frac{A_1\, a_{y_2} - A_2\, a\, y_1}{a_{x_1} a_{y_2} - a_{x_2} a_{y_1}} \qquad \text{Eq. (i)}$$

$$C_y = \frac{A_1 a x_2 - A_2 a x_1}{a y_1 a x_2 - a y_2 a x_1} \quad \text{Eq. (ii)}$$

Where, A_1 and A_2 are absorbances of mixture at λ_1 and λ_2 respectively, ax_1 and ax_2 are absorptivities of Amlodipine besylate at λ_1 and λ_2 respectively and ay_1 and ay_2 are absorptivities of Enalapril maleate at λ_1 and λ_2 respectively. C_x and C_y are concentrations of Amlodipine besylate and Enalapril maleate/Lisinopril dihydrate/Losartan potassium respectively.

At λ_1: $A_1 = a_{x1}C_x + a_{y1}C_y$ …….(1)

Atλ_2: $A_2 = a_{x2}C_x + a_{y2}C_y$ ……. (2)

5.6: Molar absorptivity and Percent molar absorptivity

The molar absorptivity and percent molar absorptivity was calculated by using formula

$\epsilon_{1\%,\ 1cm}$ = Absorbance / Concentration (g/100ml).

ϵmax = Absorbance × mol. wt. × 100/ wt (in mg) of compound in 100ml × pathlength

Preparation of stock solution

The standard stock solutions (1mg ml^{-1}) of Amlodipine besylate and Enalapril maleate were prepared by transferring 100mg (approximately but accurately weighed) of Amlodipine besylate and Enalapril maleate in 100 ml of calibrated volumetric flask separately and volume was made up to the mark with solvent. From this stock solution different dilutions were prepared ranging from 2 µg ml^{-1} to 30 µg ml^{-1}.

Determination of absorption maxima

1ml of stock solution (1000µg/ml) of Amlodipine besylate solution and Enalapril maleate was pipette out into 100ml calibrated volumetric flask separately and volume was made up to mark with methanol : 1N HCl (1:1). The final concentration of drugs was 10µg/ml. The solution was then scanned in the UV region 200–400 nm to get absorption maxima using methanol: 1N HCl (1:1) solution as blank.

Preparation of standard curve - The standard curve was prepared by measuring absorbance of various concentration of drug against blank solvent. The graph was plotted for studying the linear relationship between absorbance and concentration.

Procedure: In different 10 ml volumetric flasks 2,4,6 ……30µg/ml (1000µg/ml) solution of Amlodipine besylate solution and Enalapril maleate was placed and volume was made up to the mark with solvent. The absorbance of solution was measured at 239 nm and 209 nm respectively.

Preparation of sample solution

Twenty tablets of marketed formulation Amtas E (Intas, Ahemdabad, Gujarat, India) containing Amlodipine besylate 5 mg and Enalapril maleate 5 mg were weighed, and finely powdered. For analysis of drug, a standard addition method was used. Quantity of powder equivalent to 5 mg of Enalapril maleate and 5 mg of Amlodipine besylate was weighed and dissolved in 40 ml of methanol: 1N HCl (1:1) and sonicated for 10 minutes. Then the solution was filtered through Whatman filter paper and then final volume of the solution was made up to 100 ml with methanol: 1N HCl (1:1) to get a stock solution containing 1000µg/ ml of Amlodipine besylate and 1000µg/ ml of Enalapril maleate. In 100ml volumetric flask 2 ml of the sample solution was placed and volume was made up to the mark with solvent and absorbance was measured at 239 nm and 209 nm. The absorbance values were recorded and with the help of standard curve of both drugs, the concentration of Amlodipine besylate and Enalapril maleate in sample solution was calculated.

Chromatographic conditions for HPLC

The chromatographic separation was carried out using Analytical Technologies Ltd HPLC system with UV-2230 UV-Vis detector, P-2230 HPLC pump and a Phenomenex Gemini C18 column (250 × 4.6 mm, 5µm particle size). The isocratic mobile phase consisting of methanol: 1N HCl (1:1) and methanol: 0.1N HCl (1:1). The flow rate of the mobile phase was 1.0 ml/min. The column temperature was kept ambient and injection volume was 20µl.

Chromatographic conditions for UPLC

The separation was achieved on Waters Acquity UPLC BEH C18 column (2.1 × 50mm i.d., 1.7 µm particle size). The mobile phase methanol: 1N HCl (1:1) consisting of buffer

used throughout the analysis. The flow rate of mobile phase was 1 ml/min and the detection was monitored at UV-visible PDA detector. The mobile phase was filtered through a nylon 0.22 μm membrane filter and was degassed before use. The column temperature was maintained at 25°C and injection volume was 5 μl.

Mobile phase preparation

The mobile phase consist of methanol: 1N HCl (1:1) and methanol: 0.1N HCl (1:1) as solvent. Mobile phase was filtered through a 0.22 μm membrane filter before use and degassed in an ultrasonic bath.

Buffer preparation

4.7 g of sodium dihydrogen orthophosphate and 1 ml of triethylamine in 1000 ml of water and the pH of the solution was adjusted to 4.0± 0.05 with orthophosphoric acid.

Method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes.

The developed method for simultaneous estimation was validated as per ICH guidelines.

Parameters for method validation are listed below:

Linearity

Linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range.

Determination - To establish linearity of the proposed method, separate series of solutions were prepared from the stock solutions and analyzed. Linearity was determined by a series of five to six estimation. The responses were found directly proportional to the concentrations of the analytes.

Range

ICH defines the range of an analytical procedure as the interval from the upper to the lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Accuracy

ICH defines the accuracy of an analytical procedure as the closeness of agreement between the conventional true value or an accepted reference value and the value found.

Accuracy may be estimated from the recovery of a known standard solution ‘‘spiked’’ or added into the sample. That is, a known amount of the same substance that is to be tested is added to an aliquot of the sample, usually as a solution, prior to the analysis. The concentration of the analyte in the spiked solution of the sample is then measured. The percent spike recovery is then calculated.

$$\% \text{ Recovery} = \frac{100\ (Xs - Xu)}{K}$$

Where, **Xs**= Measured value for the spiked sample

Xu= Measured value for the unspiked sample adjusted for the dilution of the spike

K= Known value of spike in the sample.

To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 80%, 100% and 120%. known amounts of standard were added to pre-analyzed samples and were subjected to the proposed method.

Acceptance criterion

Recovery need not be 100% but all result should fall between ±20% of the expected value at lower concentration and ±15% at higher concentration and the percent relative standard deviation should be less than 2.

Precision

The precision of analysis is usually determined by running duplicate or replicate testing on one of the samples in a given batch of samples. It is expressed statistically as standard deviation, relative standard deviation (RSD), coefficient of variance (CV),

standard error of the mean (*M*), and relative percent difference (RPD). The standard deviation in measurements, however, can vary with the concentrations of the analytes. On the other hand, RSD, which is expressed as the ratio of standard deviation to the arithmetic mean of replicate analyses and is given as a percent, does not have this problem and is a more rational way of expressing precision:

$$\text{RSD} = \frac{\text{Standard deviation}}{\text{Arithmetic mean of replicate analysis}} \times 100\%$$

The precision of the method, as intra-day repeatability was evaluated by performing six independent assays of the test sample preparation and calculating the % RSD. The intermediate (interday) precision of the method was checked by performing same procedure on different days by another person under the same experimental conditions.

Robustness

The standard solution was prepared and injected for 3 times with following condition

1) Aq. Phase ratio of mobile phase has changed ± 1%
2) Flow rate has changed ± 10%
3) Change of wavelength

Limit of detection and Limit of quantification (LOD, LOQ)

The LOD and LOQ of the proposed method were determined by progressively injecting lower concentrations of the standard solutions under the set chromatographic conditions.

L.O.D. = 3.3(SD/S)

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response,

S =Slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte

- Simultaneous equation method to be performed for Amlodipine besylate and Enalapril maleate, Amlodipine besylate and Lisinopril dehydrate, Amlodipine besylate and Losartan potassium using solvent methanol : 1N HCl (1:1) and methanol : 0.1N HCl (1:1)

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